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Microsatellite Instability at Multiple Loci in Gastric Carcinoma: Clinicopathologic Implications and Prognosis

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Background & Aims: Microsatellite instability (replication error [RER]–positive phenotype) is a frequent genetic alteration in gastric carcinomas. The clinical relationship between RER-positive and RER-negative gastric tumors is poorly characterized. The aim of this study was to investigate the relationship between the number of altered microsatellite loci and the clinicopathologic features of gastric carcinoma. *Methods:* Five or 6 microsatellite loci were analyzed in 61 gastric carcinomas using polymerase chain reaction. *Results:* Twenty-one carcinomas (34.4%) had microsatellite instability: 7 at 1 locus, 2 at 2 loci, and 12 at multiple loci. The comparison between the three groups (with none, 1 or 2, and more than 2 RER-positive loci) showed that RER-negative carcinomas and carcinomas with 1 or 2 RER-positive loci share features that differ from those of carcinomas with multiple RER-positive loci. The latter were all of the intestinal or atypical subtype and had lower DNA content, more prominent lymphoid infiltration, and less prevalent nodal metastases than carcinomas in the other two groups. The patients with carcinomas showing multiple RER-positive loci had a better prognosis. *Conclusions:* The finding of microsatellite instability in a single or few loci does not qualify a case as a mutator phenotype from a clinical standpoint. Gastric tumors with multiple RER-positive loci have a particular clinicopathologic profile leading to a better outcome.

Recently, subtle genetic alterations resulting in expansions or contractions of simple repeated sequences, the so-called microsatellite instability (MI), have been described in several human cancers.[1–5] An association between the presence of MI and certain clinicopathologic characteristics was observed for sporadic colorectal carcinomas.[1–3,6,7] Lothe et al.[6] found that these differences relied on the number of altered microsatellite loci: carcinomas with multiple replication error (RER)-positive loci were significantly associated with poor differentiation, diploidy, right-sided location, and prolonged survival, whereas carcinomas with a single RER-positive locus were similar to RER-negative carcinomas.[6] MI at either one or multiple loci was also observed in other cancers, but no clinical or pathological differences were found between these two groups of tumors.[8,9]

MI has been described as a frequent genetic alteration in gastric carcinomas.[4,5,10–13] In a previous study,[14] we investigated the frequency of RER-positive phenotype in a series of 34 sporadic gastric carcinomas by analyzing 4–12 loci per case. We confirmed the frequent occurrence of the RER-positive phenotype in gastric carcinomas (32.4%) and observed that RER-positive gastric tumors tended to occur as intestinal or atypical carcinomas in the antrum of elderly patients, to have abundant T-cell infiltration, and to have a relatively good prognosis.[14]

In the aforementioned study,[14] a presence of MI at a single locus was used as a criterion for classifying a case as RER-positive. Interestingly, only 1 of the 34 carcinomas had MI in 1 locus.[14] The remaining were either RER-negative (n = 23) or had MI at ≥3 loci (n = 10), which suggested that there was a discontinuous distribution of the number of affected loci in gastric carcinomas, leading to the constitution of two groups: cases with no or very few affected loci and those with multiple affected loci. Also, we observed that the carcinoma with MI at 1 locus shared the clinicopathologic features of the RER-negative carcinomas, thus differing from the other RER-positive cases.

In the present study, we extended the previous study of gastric carcinomas by analyzing a similar number of loci in all samples to better define the clinicopathologic profile(s) of RER-positive tumors and, in particular, to find whether there is a relationship between the number of altered microsatellite loci and the clinicopathologic features of gastric carcinoma.

Abbreviations used in this paper: cCTP, deoxycytidine triphosphate; MI, microsatellite instability; PCR, polymerase chain reaction; RER, replication error; WHO, World Health Organization.

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Materials and Methods

Patients, Tissue Samples, and DNA Extraction

We analyzed 61 gastric carcinomas and paired samples from nonneoplastic mucosa of white patients who underwent surgery at the Hospital S. João, Oporto, Portugal. Thirty-four of the 61 cases have been reported previously.[14] None of the patients included in the present series had a family history suggestive of hereditary nonpolyposis colorectal cancer. H&E-stained sections were used to categorize tumors according to the classifications of the World Health Organization (WHO),[15] Laurén,[16] and Ming.[17] Penetration of the gastric wall and the presence and localization of lymph node metastases were recorded for all patients using standard criteria for pathological staging. Orcein-stained sections were used for the detection of vascular invasion. Lymphoid infiltration was subjectively scored as absent/minimal or moderate/abundant. Immunohistochemistry was used to classify the lymphocytic infiltration of the tumors according to the predominance of T or B lymphocytes using UCHL1 and L26 antibodies (Dako, Glostrup, Denmark), respectively. Ploidy was determined by flow cytometry in frozen samples as previously described.[18]

From each patient, tissue fragments from primary tumors and nonneoplastic mucosa were immediately frozen in liquid nitrogen and stored at −70°C until use. The percentage of nonmalignant "contaminating" cells in the tumor samples was estimated by scoring adjacent histological cryostat sections. Microdissection of the specimens was performed whenever necessary. Only samples with more than 50% of tumor cells were included in the study. High-molecular-weight DNA was isolated using standard methods.[19]

Microsatellite Marker Analysis

Six loci containing dinucleotide repeat sequences were studied: D1S158, D2S104, D5S82, D5S346, D6S252, and ARG1. Primers specific for each locus were used to amplify the repeat and short flanking sequences in template DNA using polymerase chain reaction (PCR). PCR was performed ppin a 25-μL volume of a mixture containing 10 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 100 μmol/L of each deoxynucleotide triphosphate except deoxycytidine triphosphate (dCTP), 10 μmol/L dCTP, 1 μCi [α-^{32}P]dCTP, 0.4 μmol/L of each primer, 0.75 U *Taq* DNA polymerase, and 30–50 ng genomic DNA. Thirty cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1.75 minutes were performed with an initial denaturation step of 94°C for 5 minutes and a final extension step of 72°C for 10 minutes using a Perkin–Elmer 9600 GeneAmp PCR System (Perkin–Elmer, Norwalk, CT). PCR reaction products were diluted 1:1 with a loading buffer (98% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 10 mmol/L ethylenediaminetetraacetic acid [pH 8.0]) and then denatured for 5 minutes at 95°C. Subsequently, 2 μL of this solution was electrophoresed in 6% polyacrylamide gels containing 6.9 mol/L urea and 32.5% formamide for 2–3 hours at 60 W. After electrophoresis, gels were fixed in 10% acetic acid, washed, dried, and exposed to x-ray film for 12–24 hours. All scoring was performed independently by two observers. Five or 6 loci were analyzed per case. Of the 61 tumors, 16 tumors were analyzed at 5 loci and 45 tumors were analyzed at 6 loci. A case was considered positive when at least 1 locus had a different mobility band. All RER-positive loci were confirmed by a new PCR and electrophoretic run.

Statistical Analysis

Statistical analysis was performed using the χ^2 test with Yates' correction, Fisher's Exact Test, or Student's *t* test using Statview 4.01 software. Follow-up data were obtained in all but 1 patient. Survival curves were calculated according to Berkson's actuarial method and compared using the generalized Wilcoxon (Breslow) test using BMDP (PC-92) software (BMDP Statistical Software, Cork, Ireland). A P value of <0.05 was considered statistically significant, and a P value of <0.1 was considered suggestively significant.

Results

Twenty-one of 61 carcinomas (34.4%) had MI (Figure 1). Of the 21 RER-positive carcinomas, 7, 2, 2, 5, 4, and 1 had MI at 1, 2, 3, 4, 5, and 6 loci, respectively. Of the 7 tumors that showed only 1 affected microsatellite, 6 were analyzed at 6 loci and 1 was analyzed at 5 loci. The 2 tumors that showed 2 affected microsatellites were analyzed at 6 loci. Of the 12 tumors with MI at ≥3 or more loci (multiple loci), 2 were analyzed at 5 loci and 10 were analyzed at 6 loci. Both microsatellite expansions and contractions were identified independently of the number of altered loci.

After having verified that the 2 carcinomas with RER-positive at 2 loci had clinicopathologic features almost identical to those of carcinomas with 1 RER-positive locus, we divided the RER-positive carcinomas in two groups: a group of 9 carcinomas with 1 or 2 RER-positive loci and a group of 12 carcinomas with multiple RER-positive loci (Table 1). Clinical and pathological data of the gastric carcinomas are shown in Table 1. Because we perceived that most clinicopathologic features of carcinomas with 1 or 2 RER-positive loci were similar to those of RER-negative carcinomas (Table 1) and different from those of carcinomas with multiple RER-positive loci, we joined the former two groups of carcinomas and compared them with the latter (Table 2).

According to Laurén's classification, none of the carcinomas with multiple RER-positive loci were of the diffuse type, in contrast to 35% carcinomas with none or 1 or 2 RER-positive loci ($P = 0.06$). These differences were also noted for histological typing according to WHO classification because no signet ring cell or mucinous gastric carcinoma had MI at more than 2 loci ($P < 0.1$). At variance with carcinomas with none or 1

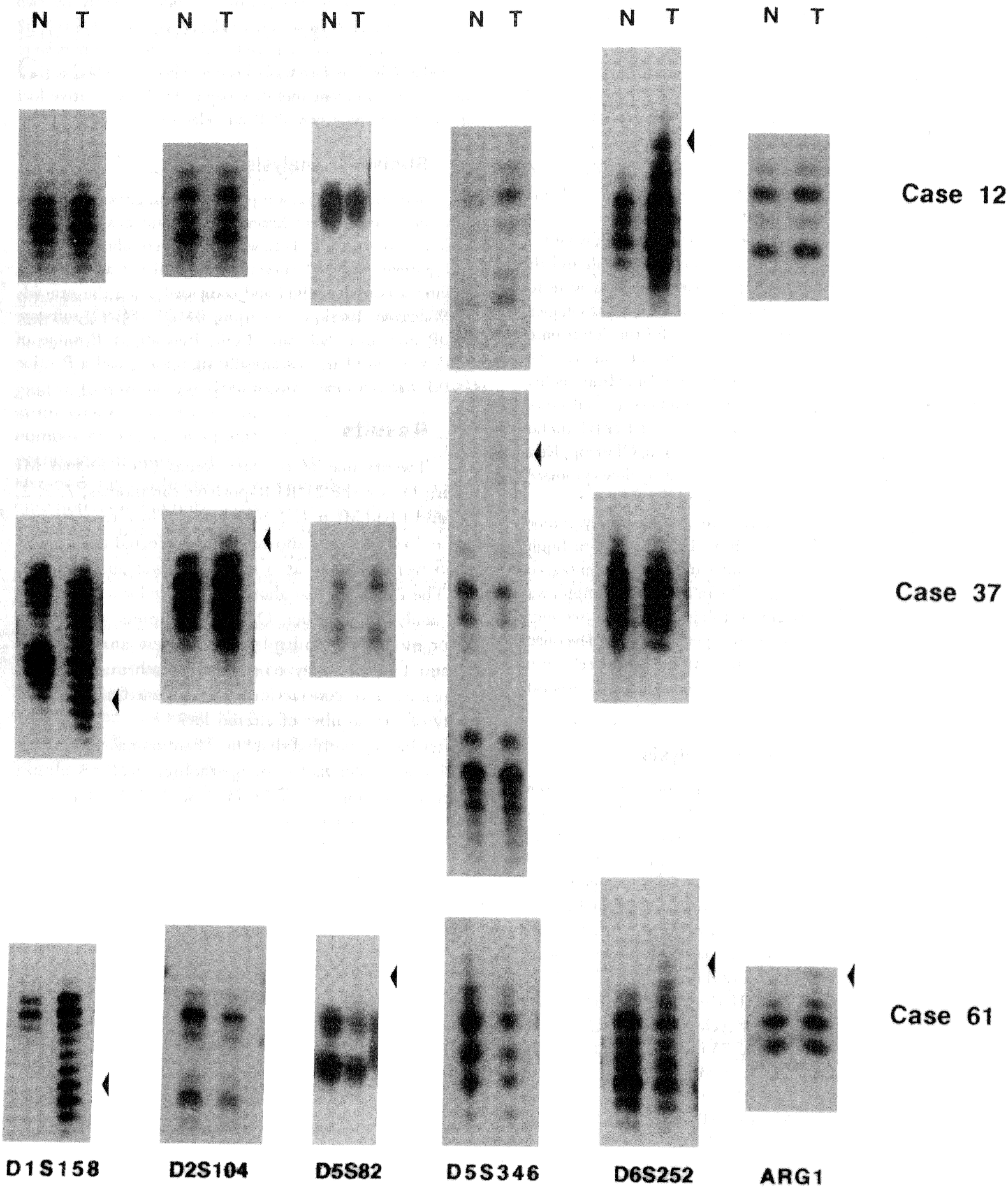

Figure 1. MI in gastric carcinomas using 6 microsatellite loci (D1S158, D2S104, D5S82, D5S346, D6S252, and ARG1). Case 12 has 1 locus with instability (D6S252), case 37 has 3 loci with instability (D1S158, D2S104, and D5S346), and case 61 has 4 loci with instability (D1S158, D5S82, D6S252, and ARG1). N, normal DNA; T, tumoral DNA; *arrowheads*, new bands.

Table 1. Relation Between MI (RER-Positive Phenotype) and Clinicopathologic Characteristics of the Gastric Carcinoma Cases

Clinicopathologic characteristics	Number of cases with (+) or without (−) RER				
	RER-negative ($n = 40$)	RER-positive at 1 or 2 loci ($n = 9$)	RER-positive at ≥3 loci ($n = 12$)	Total ($n = 61$)	*P* value[a]
Age (mean ± SD)	59.2 ± 12.9	62.7 ± 12.5	63.8 ± 10.6	61.5 ± 12.1	NS
Male/female	1.7/1	3.5/1	1/1	0.7/1	NS
Location[b]					
Antrum	20	6	8	34	
Body	14	2	3	19	
Cardia	6	1	0	7	NS
Gross type					
Vegetative	12	0	6	18	
Ulcerated	13	5	4	22	
Infiltrating	14	4	2	20	NS
Size ± SD (*cm*)	7.7 ± 4.9	8.5 ± 8.9	10.3 ± 4.6	8.1 ± 5.3	NS
Classification of Laurén[16]					
Intestinal	14	4	7	25	
Diffuse	14	3	0	17	
Atypical	12	2	5	19	NS
Classification of WHO[c]					
Well-differentiated	14	4	8	26	
Poorly differentiated	12	2	4	18	
Signet-ring cell	12	3	0	15	
Mucinous	2	0	0	2	NS
Classification of Ming[17]					
Expanding	13	4	8	25	
Infiltrative	27	5	4	36	NS
Lymphoid infiltrate					
Minimal/absent	31	7	4	42	
Moderate/abundant	9	2	8	19	0.012
Desmoplastic response					
Minimal/absent	25	5	12	42	
Moderate/abundant	15	4	0	19	0.031
Vascular invasion					
Absent	16	5	6	27	
Present	24	4	6	34	NS
Wall invasion					
Within the gastric wall	32	7	9	48	
Invading the serosa	8	2	3	13	NS
Lymph node metastases					
Absent	7	4	6	17	
Present	33	5	6	44	0.043
DNA index ± SD[d]	1.49 ± 0.48	1.42 ± 0.36	1.12 ± 0.20	1.42 ± 0.48	0.012
S-phase fraction ± SD[d]	20.3 ± 8.2	23.4 ± 13.4	17.4 ± 3.4	20.0 ± 8.6	NS

[a] *P* value was obtained using χ^2 test with Yates' correction and Student's *t* test (NS = $P \geq 0.01$).
[b] In 1 patient, location was not determined.
[c] Simplified version of WHO classification.
[d] In 10 cases, DNA index and S-phase fraction were not determined.

or 2 RER-positive loci, carcinomas with multiple RER-positive loci showed predominantly expanding growth pattern ($P = 0.06$) and always had minimal or absent desmoplastic response ($P < 0.05$) (Table 2).

Statistically significant differences were also observed regarding lymphoid infiltrate ($P < 0.01$) and DNA content ($P < 0.005$) (Table 2). Sixty-seven percent of gastric carcinomas with more than 2 RER-positive loci had moderate or abundant lymphoid infiltrate in contrast to only 22% of carcinomas with none or few RER-positive loci. Lymphoid infiltration was mainly composed of T cells, as assessed by immunohistochemistry (data not shown). All carcinomas with MI at multiple loci were diploid or near diploid (average DNA index, 1.04 ± 0.07), and most carcinomas without MI or with 1 or 2 affected loci had aneuploid DNA content (average DNA index, 1.48 ± 0.46).

The percentage of cases with lymph node metastases was lower ($P = 0.08$) in the group of carcinomas with multiple RER-positive loci (50%) than in the other group of carcinomas (78%).

Table 2. Significant Clinicopathologic Differences Between Gastric Carcinomas With MI at Multiple Loci and Carcinomas With MI at None, One, or Two Loci

Clinicopathologic characteristics	No. of cases with (+) or without (−) RER			
	RER-positive at ≤2 loci (n = 49)	RER-positive at ≥3 loci (n = 12)	Total (n = 61)	*P* value[a]
Classification of Laurén[16]				
Intestinal	18	7	25	
Diffuse	17	0	17	
Atypical	14	5	19	0.055
Classification of WHO[b]				
Well-differentiated	18	8	26	
Poorly differentiated	14	4	18	
Signet-ring cell	15	0	15	
Mucinous	2	0	2	0.099
Classification of Ming[17]				
Expanding	17	8	25	
Infiltrative	32	4	36	0.091
Lymphoid infiltrate				
Minimal/absent	38	4	42	
Moderate/abundant	11	8	19	0.009
Desmoplastic response				
Minimal/absent	30	12	42	
Moderate/abundant	19	0	19	0.024
Lymph node metastases				
Absent	11	6	17	
Present	38	6	44	0.056
DNA index ± SD[c]	1.48 ± 0.46	1.04 ± 0.07	1.42 ± 0.48	0.003

[a]*P* value was obtained using χ^2 test with Yates' correction and Fisher's Exact Test.
[b]Simplified version of WHO classification.
[c]In 10 cases, DNA index was not determined.

Univariate analysis showed that patients with carcinomas with MI at multiple loci had significantly better survival ($P < 0.05$) than patients with carcinomas with no RER or with 1 or 2 RER-positive loci (Figure 2). Multivariate analysis was not performed because of the small size of the sample and the short follow-up time in most patients (median follow-up, 3 years).

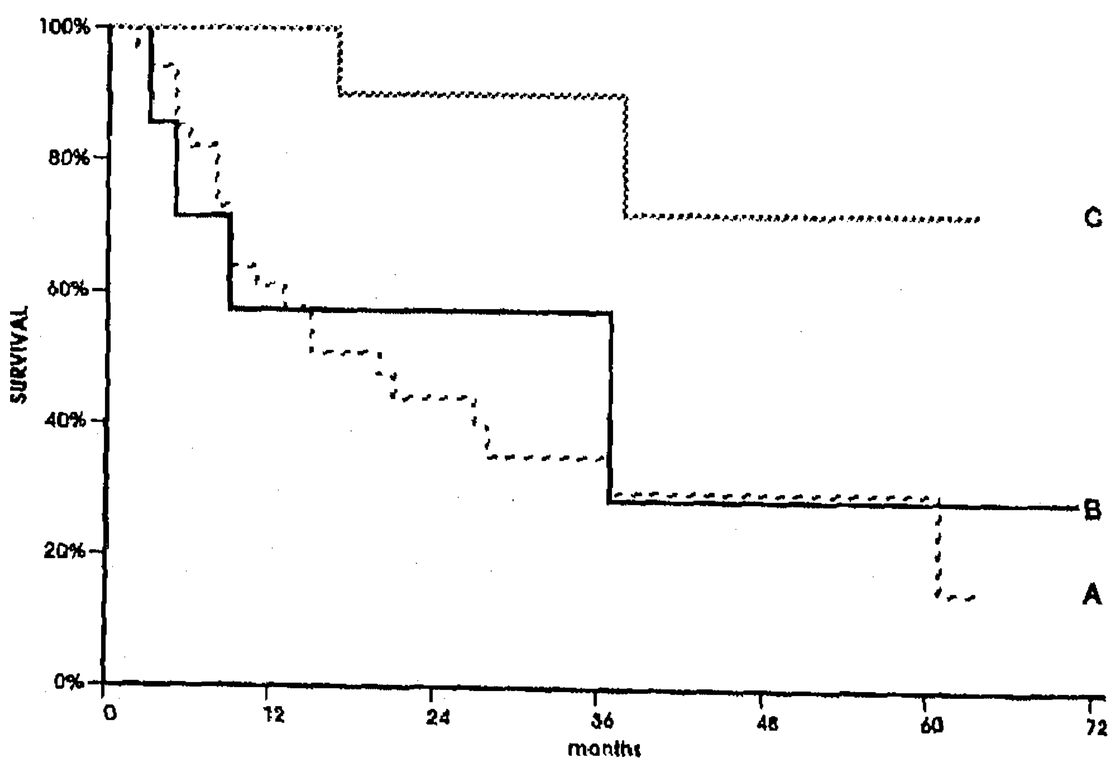

Figure 2. Cause-specific survival (death from gastric cancer; Berkson's actuarial method) for 60 patients with relation to MI. A, carcinomas with no MI (n = 39); B, carcinomas with 1 or 2 RER$^+$ loci (n = 9); C, carcinomas with multiple RER-positive loci (n = 12). The comparison of C vs. A and B yields a statistically significant difference ($P = 0.026$) using the generalized Wilcoxon (Breslow) test.

Discussion

In a previous study, we detected MI in 32.4% of 34 cases of gastric carcinomas[14] and found that these RER-positive carcinomas were associated with some clinicopathologic features, as previously reported in studies on colorectal carcinomas.[1–3,6,7]

In the present study, we extended the previous study to a total of 61 patients and detected 21 gastric carcinomas with MI (34.4%). Of these 21 RER-positive carcinomas, 9 had MI instability at 1 (n = 7) or 2 loci (n = 2) and 12 had MI at multiple loci. After comparing the clinicopathologic features of the three groups of carcinomas (RER-negative, RER-positive at few loci, and RER-positive at multiple loci), we found that carcinomas with none or 1 or 2 RER-positive loci share several features that are different from those of carcinomas with multiple RER-positive loci. These observations prompted us to compare carcinomas with multiple RER-positive loci with the remaining carcinomas, leading to the finding of an association between the presence of multiple RER-positive loci and intestinal/atypical histological differentiation, expanding growth pattern, absent or minimal

desmoplastic response, abundant lymphoid infiltration, low DNA content, relatively low frequency of lymph node metastization, and better prognosis.

At variance with our previous study[14] in which we classified every carcinoma having MI as a mutator phenotype regardless of the number of affected loci, our present results indicate that MI is only associated with a particular set of clinicopathologic features when it occurs at multiple loci, as was previously shown by Lothe et al.[6] in a study of colorectal carcinomas. Our results, similar to those of Thibodeau et al.[2] and Lothe et al.,[6] therefore show that one cannot rely on the finding of a single or a few RER-positive loci to consider a gastrointestinal carcinoma as being a mutator phenotype from a clinicopathologic standpoint. It is likely that the drawing line distinguishing "positive" from "negative" cases will depend on the number of loci analyzed and on the number and characteristics of the markers used in each study.

At least three hypotheses can explain the clinicopathologic disparity between carcinomas with few RER-positive loci and carcinomas with multiple RER-positive loci. First, the occurrence of MI at few loci may represent a random event or an abnormality of one of the DNA polymerases, whereas the occurrence of MI at multiple loci may indicate abnormalities of DNA mismatch repair genes. Second, mutations in different mismatch repair genes or in different domains of those genes can lead to a variable degree of hypermutability.[20] Carcinomas with a few RER-positive loci may carry a distinct mismatch repair deficiency compared with carcinomas with multiple RER-positive loci, therefore conferring to the former characteristics similar to those of RER-negative carcinomas. In agreement with this possibility, Bhattacharyya et al.[21] reported colorectal carcinoma cell lines showing quite different rates of MI. Third, inactivating mutations in mismatch repair genes resulting in mismatch repair deficiency may occur at different steps during tumor progression. Carcinomas with 1 or 2 RER-positive loci may have acquired the RER-positive phenotype in an advanced phase of tumor progression, therefore not giving enough time to yield distinctive clinicopathologic features. In contrast, carcinomas with MI at multiple loci would have acquired a mismatch repair deficiency in earlier steps of tumor progression, thus leading to the accumulation of multiple mutations throughout the genome, including microsatellite and gene sequences.

It is difficult or even impossible to evaluate the plausibility of the aforementioned hypotheses without knowing what is going on at the different mismatch repair genes that may be involved in generating MI in gastric carcinomas. However, if one takes in consideration that carcinomas with "few" RER-positive loci are mostly aneuploid and clinicopathologically indistinguishable from RER-negative carcinomas, it seems likely that the generation of MI in these tumors is just part of the bulk of secondary genetic changes related to tumor progression.

All carcinomas with multiple RER-positive were classified as intestinal or atypical gastric carcinomas in keeping with previous reports.[10–12] The low frequency of MI in diffuse carcinomas does not seem to be an underrepresentation caused by stromal contamination because, in the present study, we only included study samples with more than 50% of tumor cells. We also observed associations between the presence of the RER-positive phenotype and the occurrence of minimal or absent desmoplastic response ($P < 0.05$) and expanding growth pattern ($P = 0.09$). These associations are not unexpected because the aforementioned features are also strongly associated ($P = 0.001$, χ^2 test) with the intestinal and atypical types of gastric carcinoma. We verified that no cardiac carcinoma had MI at multiple loci, thus confirming that mutator phenotype cancers have particular organ locations, although this had no statistical significance.[1,6,7,11,14]

We observed a significant association between RER-positive phenotype and low (diploid or near diploid) DNA content, as previously reported for colorectal carcinomas.[6] These findings point to the possibility that the RER-positive mutator phenotype and the gross genetic alterations associated with aneuploidy may be each one part of two alternative pathways in tumor development, as previously suggested by Chong et al.[10] and Shibata et al.[22] On the other hand, the frequent acquisition of a mismatch repair deficiency, manifested as MI in intestinal and atypical gastric carcinomas and not in diffuse carcinomas, supports the existence of two carcinogenic pathways for gastric carcinoma, one leading to the intestinal type and the other to the diffuse type.[23,24]

Our finding of an association between the presence of multiple RER-positive loci and the lower prevalence of lymph node metastization is in accordance with the negative correlation found between MI and metastization, previously reported in gastric carcinomas[11] and colorectal carcinomas.[1] The low prevalence of lymph node metastization most likely accounts for the good outcome of the patients with carcinomas with multiple RER-positive loci, as previously reported for colorectal cancer.[6]

The statistically significant association between the presence of MI and moderate/abundant lymphoid infiltration confirms our previous results[14] and, together with the presence of Crohn's-like lymphoid reaction in colorectal RER-positive carcinomas,[7] supports the assumption that unrepaired errors in certain genes can lead to the appearance of new surface molecules and thereby trigger an immune response.[25] This mechanism may ac-

count for the better prognosis of patients with carcinomas with multiple RER-positive loci if one assumes that the immune response may limit the invasive and/or metastatic ability of the tumors.

The mutator phenotype may also contribute for the better clinical outcome of patients with multiple RER-positive loci through the acquisition of a mutation burden, which can yield deleterious mutations in vital genes.[22,25] A mutational load may negatively condition the selective advantage conferred by favorable mutations in cancer-related genes, therefore leading to an indolent tumoral progression and low metastatic potential. This scenario is in agreement with the lower proliferative index of carcinomas and older age of patients with multiple RER-positive loci.

In summary, the finding of MI in a single or few loci does not qualify a gastric cancer case as a mutator phenotype from a clinical standpoint, whereas gastric carcinomas with multiple RER-positive loci have a particular clinicopathologic profile leading to a better outcome.

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